

Pancreatic Stellate Cells: Partners in Crime with Pancreatic Cancer Cells

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Abstract

Pancreatic stellate cells (PSC) produce the stromal reaction in pancreatic cancer, but their role in cancer progression is not fully elucidated. We examined the influence of PSCs on pancreatic cancer growth using (a) an orthotopic model of pancreatic cancer and (b) cultured human PSCs (hPSC) and human pancreatic cancer cell lines MiaPaCa-2 and Panc-1. Athymic mice received an intrapancreatic injection of saline, hPSCs, MiaPaCa-2 cells, or hPSCs + MiaPaCa-2. After 7 weeks, tumor size, metastases, and tumor histology were assessed. *In vitro* studies assessed the effect of cancer cell secretions on PSC migration and the effect of hPSC secretions on cancer cell proliferation, apoptosis, and migration. Possible mediators of the effects of hPSC secretions on cancer cell proliferation were examined using neutralizing antibodies. Compared with mice receiving MiaPaCa-2 cells alone, mice injected with hPSCs + MiaPaCa-2 exhibited (a) increased tumor size and regional and distant metastasis, (b) fibrotic bands (desmoplasia) containing activated PSCs within tumors, and (c) increased tumor cell numbers. *In vitro* studies showed that, in the presence of pancreatic cancer cells, PSC migration was significantly increased. Furthermore, hPSC secretions induced the proliferation and migration, but inhibited the apoptosis, of MiaPaCa-2 and Panc-1 cells. The proliferative effect of hPSC secretions on pancreatic cancer cells was inhibited in the presence of neutralizing antibody to platelet-derived growth factor. Our studies indicate a significant interaction between pancreatic cancer cells and stromal cells (PSCs) and imply that pancreatic cancer cells recruit stromal cells to establish an environment that promotes cancer progression. [Cancer Res 2008;68(7):2085–93]

Introduction

Pancreatic cancer has a very poor prognosis largely due to its propensity for early local and distant invasion (1–4). In recent years, researchers studying the pathogenesis of this disease have turned their attention to the desmoplastic/stromal reaction, a characteristic feature of the majority of pancreatic cancers (5, 6). This reaction consists of abundant fibrous tissue composed of extracellular matrix (ECM) proteins, new blood vessels, and stromal cells (7). It is possible that interactions between stromal

cells and tumor cells influence the progression of the disease. This concept is supported by findings from studies on breast cancer (8, 9) and prostate cancer (10, 11), which also exhibit a significant stromal component.

Work by our group and those of others have established that the cells responsible for production of the desmoplastic reaction in pancreatic cancer are pancreatic stellate cells (PSC; refs. 5, 6, 12), which are now recognized as key cells in pancreatic fibrogenesis (6, 13–17). Activation of PSCs by growth factors, cytokines, and oxidant stress results in their transformation from a quiescent to a myofibroblast-like phenotype, which secretes excess amounts of ECM proteins (6, 15, 17–20). Given that pancreatic cancer cells secrete numerous growth factors such as transforming growth factor- β 1 (TGF- β 1), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF; ref. 21), all of which are known to activate PSCs, it is not surprising that *in vitro* studies have shown activation of PSCs exposed to pancreatic cancer cell secretions. Using neutralizing antibodies, Bachem et al. (6) have reported that the inductive effect of pancreatic cancer cells on ECM protein synthesis by PSCs may be mediated by TGF- β 1 and basic fibroblast growth factor (bFGF).

To determine whether the observed *in vitro* interactions between tumor cells and PSCs are relevant to the *in vivo* situation, researchers have turned to animal models of pancreatic cancer. Using s.c. xenografts of a human pancreatic cancer cell line in nude mice, Bachem et al. (6) have shown that the rate of tumor growth in mice injected with a mixture of cancer cells and PSCs was significantly greater than that in mice injected with cancer cells alone. S.c. tumor models provide useful data but are somewhat limited because they do not allow an assessment of tumor behavior within the organ of interest and also cannot be used to assess processes such as distant metastasis of the cancers under study. These limitations can be largely overcome by using orthotopic models of cancers.

Most of the orthotopic models of pancreatic cancer described in the literature have only assessed tumors produced after injection of pancreatic cancer cells alone (22–24). Even where pancreatic tumors were produced in nude mouse pancreas by implantation of small pieces of human pancreatic cancer tissue, the stromal component of pancreatic cancers has largely been ignored. Therefore, the aim of this work was to examine tumor growth, local invasion, and distant metastasis using an orthotopic model of pancreatic cancer produced by injection of a suspension of pancreatic cancer cells with and without PSCs into the tail of the pancreas of nude mice. To our knowledge, these are the first studies to assess the interactions between tumor cells and the stromal component of pancreatic cancer in a physiologically representative *in vivo* situation. The work described in this article also includes *in vitro* findings related to the direct effects of PSCs on pancreatic cancer cells. Our results indicate that the interaction between these

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two cell types is not merely unidirectional (cancer cells influencing PSCs) but also bidirectional because PSCs were also found to significantly influence cancer cell survival (by increasing proliferation and, at the same time, inhibiting apoptosis of the latter).

Materials and Methods

Reagents. The reagents used were Xten Hybricell SFM medium (Thermo Electron Corporation); Iscove's modified Dulbecco's medium, and fetal bovine serum (FBS; Invitrogen Pty. Ltd.); Enrofloxacin (Bayer Australia Ltd.); anti- α -smooth muscle actin (α SMA) antibody (Sigma); antihuman nuclear antigen antibody (Chemicon); anti-proliferating cell nuclear antigen (PCNA) antibody, 3,3-diaminobenzidine tetrahydrochloride substrate chromogen, mouse monoclonal anti-cytokeratin, and mouse monoclonal anti-GFAP (DAKO); [3 H]thymidine (ICN); antihuman PDGF receptor β , antihuman PDGF-BB, antihuman bFGF, and anti-TGF- β 1 antibody (R&D Systems, Bio-Scientific); and TdT-FragEL DNA Fragmentation Detection Kit (Calbiochem). Cell Culture Inserts (8 μ m pores) were from Becton Dickinson S.A., and Cultrex Matrigel Invasion Assay was from Trevigen, Inc.

PSC Isolation and Culture

Human PSCs. Human PSCs (hPSC) were isolated from pancreatic tissues obtained from patients undergoing pancreatic resection using the outgrowth method as described by Bachem et al. (14). Before use, purity of PSCs was assessed by morphology and by immunostaining for the activation marker α SMA. All PSC preparations were used between the first and third passages.

Conditioned medium from hPSCs was collected after incubation of the cells for 24 h at 37°C in Xten Hybricell SFM medium containing 10% FBS. In preparation for injection in the orthotopic model, cultured hPSCs were harvested, washed, and adjusted to a cell density of $1 \times 10^6/50 \mu\text{L}$ of PBS.

Rat PSCs. Rat PSCs were isolated by density gradient centrifugation and cultured as previously described by us (25). Cells were used between the first and third passages.

Human Pancreatic Cancer Cell Lines

The human pancreatic cancer cell lines MiaPaCa-2 and Panc-1 (obtained from American Type Culture Collection) were cultured according to the supplier's instructions.

In vivo Model of Orthotopic Pancreatic Cancer

Six- to eight-week-old female athymic nude mice (BALB/c *nu/nu*) were grouped into 10 sets of four mice each. The antibiotic Enrofloxacin was administered daily by s.c. injection (5 mg/kg) for 2 d before surgery. [Note: The surgical set up was designed such that we operated on one set of four mice at a time. This was necessitated by the fact that hPSCs from resected pancreatic specimens were used in our study and we had to ensure that adequate numbers of cells were available (from each specimen) so that hPSCs from the same source could be injected either by themselves (hPSCs alone) or with MiaPaCa-2 cells (hPSCs + MiaPaCa-2) per set of four mice. This enabled a valid comparison of tumor size and other parameters within each set. Thus, within each set, paired comparisons could be made between the mouse injected with MiaPaCa-2 alone and the mouse injected with MiaPaCa-2 + hPSCs.] On the day of surgery, mice were anesthetized. An incision was made in the left flank and the spleen and tail of the pancreas were exteriorized. The four mice in each set were injected into the tail of the pancreas with one of the following: (a) 50 μL of PBS; (b) 1×10^6 hPSCs/50 μL of PBS; (c) 1×10^6 MiaPaCa-2 cells/50 μL of PBS; or (d) mixture of MiaPaCa-2 cells (1×10^6) + hPSCs (1×10^6) in 50 μL of PBS. Seven weeks after surgery, mice were sacrificed, the pancreas removed, and tumor size measured [tumor volume was calculated according to an established formula ($1/2 \text{ length} \times \text{breadth} \times \text{width}$)]. Tumors were processed for histologic examination. The abdominal cavity, mesentery, spleen, and liver were examined for the presence of metastases.

Histologic Assessment

Pancreatic sections (4–5 μm) were stained with H&E, Masson's trichrome for connective tissue, Sirius Red for collagen, and terminal deoxyribonu-

cleotidyl transferase-mediated dUTP nick end labeling (TUNEL) for apoptosis using established methods (26, 27). Sections were also immunostained for (a) α SMA (for activated PSCs); (b) cytokeratin (for tumor cells); and (c) PCNA to assess cell proliferation. In addition, serial sections of tumors and metastatic nodules in the liver were immunostained for α SMA and human nuclear antigen to differentiate between hPSCs and host (mouse) PSCs.

Immunostaining for α SMA, cytokeratin, PCNA, and human nuclear antigen. Pancreatic sections were incubated with the appropriate primary antibody: monoclonal mouse anti- α SMA antibody (1:100) for 1 h at room temperature; mouse monoclonal anti-cytokeratin (1:75) for 1 h at room temperature; monoclonal mouse anti-PCNA (1:200) overnight at 4°C; or monoclonal mouse anti-human nuclear antigen antibody (1:30) for 90 min at room temperature. Sections were then incubated with the secondary antibody horseradish peroxidase-labeled goat anti-mouse IgG (1:200) for 30 min at room temperature. Negative controls were sections incubated with mouse monoclonal isotype IgG2a (α SMA) or IgG1 (cytokeratin, PCNA, nuclear antigen).

TUNEL staining. TUNEL staining of paraffin-embedded tumor sections was done with the TdT-FragEL DNA Fragmentation Kit (Calbiochem) according to the manufacturer's instructions.

Morphometric analysis. For each of the stains described below, 10 randomly selected areas for each pancreatic section (60 \times objective, Olympus Microscope BX50, Digital Camera DP70) were photographed and then analyzed morphometrically by two separate observers blinded to sample identity.

α SMA and Masson's trichrome. The area of α SMA or Masson's trichrome positive regions per section was determined by computer-assisted morphometry (Metamorph 7.0, Molecular Devices). Briefly, pictures were loaded individually onto the software interface and a color range for brown-stained (α SMA) and blue-stained areas (ECM in Masson's trichrome staining) selected in a semiautomated fashion by the operator. The Metamorph software enabled calculation and expression of the extent of staining as a percentage of the total analyzed area and means were calculated of the 10 analyzed photomicrographs per animal.

Cytokeratin. Cytokeratin-positive cells were counted on 10 photomicrographs per specimen and results expressed as mean number of cells per square millimeter.

PCNA and TUNEL. The number of PCNA-stained and TUNEL-positive cells was assessed by grid point counting. Briefly, photomicrographs were inserted into a PowerPoint File and a grid comprising 117 points of intersection ("grid points") was overlaid onto each picture. PCNA- or TUNEL-positive cells coinciding with a grid point were counted and expressed as a percentage of total grid points. Means of data from photomicrographs of 10 randomly selected areas per section were calculated.

In vitro Studies: Interaction between PSCs and Pancreatic Cancer Cells

Effect of cancer cells on PSC migration. MiaPaCa-2 or Panc-1 cells were seeded into the wells of modified Boyden chambers (21,000 and 32,000 cells per well, respectively). Culture inserts (Becton-Dickinson) with a porous membrane at the bottom (8- μm pores) were seeded with rat PSCs (30×10^3 per insert) and then placed into the wells containing either MiaPaCa-2 or Panc-1 cells or culture medium alone (controls). PSCs and cancer cells were thus cocultured for 24 h. Migration of PSCs was then assessed as previously described by us (28, 29). The rate of PSC migration was expressed as a migration index (%): (number of cells on the undersurface of the membrane / total number of cells on both surfaces of the membrane) $\times 100$.

Effect of PSCs on cancer cell migration. Human PSCs were seeded into the wells of modified Boyden chambers (30,000 per well). Culture inserts (Becton-Dickinson) with a porous membrane at the bottom (8- μm pores) were seeded with Panc-1 cells (14×10^3 per insert) or MiaPaCa-2 cells (7×10^3 per insert) and placed into the wells containing hPSCs or culture medium alone (controls). PSCs and cancer cells were then cocultured for 48 h. Migration of cancer cells was assessed as described above for PSCs and expressed as a migration index.

Effect of PSCs on cancer cell invasion. Invasion of MiaPaCa-2 cells exposed to hPSC secretions or culture medium alone was done using the Cultrex Matrigel Cell Invasion Assay. Briefly, MiaPaCa-2 cells (5,000 per well) were seeded into basement membrane extract-coated porous culture inserts and allowed to migrate in the presence of hPSC secretions (collected in 0.1% medium) or control medium (both concentrated $\times 2$).

Effect of PSCs on pancreatic cancer cell proliferation. Panc-1 and MiaPaCa-2 cells were incubated for 24 h at 37°C with conditioned media from hPSCs obtained from five different patients. Control incubations were done with medium containing 10% FBS. Cancer cell proliferation was then assessed by measurement of incorporation of [3 H]thymidine into cellular DNA as previously published (15).

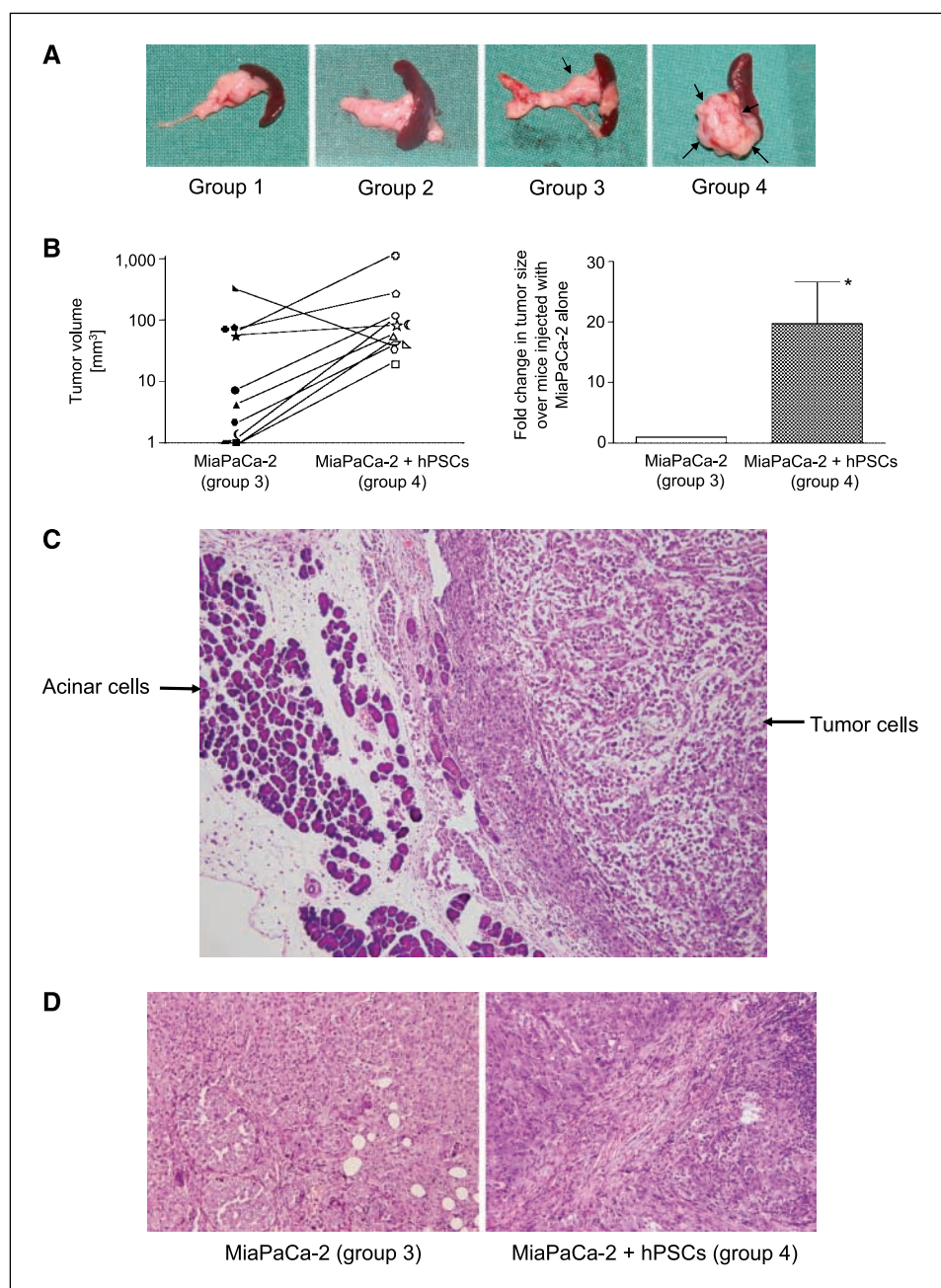
Assessment of mediators of PSC secretion-induced cancer cell proliferation. MiaPaCa-2 cells were incubated with conditioned medium from PSCs in the presence of neutralizing antibodies to known mitogenic factors including PDGF, bFGF, and TGF- β 1. Antibodies (10 μ g/mL each)

were added to the conditioned medium and incubated for 1 h at 37°C before addition to the wells containing cancer cells. After a further 8 h, cancer cell proliferation was assessed by measuring [3 H]thymidine incorporation into cellular DNA. Cells incubated with neutralizing antibody alone (in the absence of secretions) or in medium without antibodies served as controls.

Immunofluorescence for PDGF receptor β (MiaPaCa-2 cells) and PDGF-BB (PSCs). PSCs and MiaPaCa-2 cells cultured on coverslips were incubated with goat anti-human PDGF-BB antibody (1:50) or goat anti-human PDGF receptor β antibody (1:50), respectively, for 12 h at 4°C in blocking buffer. Alexa Fluor 488 rabbit anti-goat IgG (1:1,000) was used as a secondary antibody. For negative controls, purified goat IgGs were used at the same concentration as the primary antibodies.

Effect of PSCs on pancreatic cancer cell apoptosis. MiaPaCa-2 and Panc-1 cells were incubated for 7 h at 37°C with one of the following: (a) culture medium alone; (b) culture medium with H $_2$ O $_2$ (3 μ mol), a known

Figure 1. A, macroscopic appearance of representative pancreas specimens at autopsy. Pancreas of mice injected with PBS (group 1) and hPSCs (group 2) are of normal appearance. A small tumor (arrow) is visible in the pancreatic tail of the mouse injected with MiaPaCa-2 cells alone (group 3), whereas a significantly larger tumor (arrows) is seen in the pancreas of a mouse injected with MiaPaCa-2 + hPSCs (group 4). B, effect of hPSCs on tumor size. Left, tumor volumes in group 3 (MiaPaCa-2 alone) and group 4 (MiaPaCa-2 + hPSCs) mice. The corresponding pairs within each set are denoted by closed (group 3) and open (group 4) symbols and connected by a line. Right, columns, mean fold change in tumor volume of group 4 mice over group 3 mice; bars, SE ($n = 10$ mice per group). *, $P < 0.005$. C, a representative H&E-stained section of a pancreatic tumor produced by injection of a suspension of MiaPaCa-2 + hPSCs into the tail of the pancreas, showing poorly differentiated tumor cells surrounded by normal host (mouse) pancreatic acinar cells, confirming the orthotopic nature of the tumor (original magnification, $\times 200$). D, representative H&E-stained sections of pancreatic tumors from mice injected with MiaPaCa-2 cells alone (group 3) or MiaPaCa-2 + hPSCs (group 4). Tumor cells are evident in both sections. Importantly, the group 4 tumor section shows clearly demarcated bands of fibrosis (stromal reaction) within tumor tissue (original magnification, $\times 200$).



inducer of apoptosis; (c) PSC secretions; or (d) PSC secretions + H₂O₂ (3 μ mol). Apoptosis was assessed by TUNEL staining (as described earlier) and also by flow cytometry of Annexin V/propidium iodide-stained cells (30). Positively stained cells were identified and data expressed as percent of total cells.

All experiments described in this study were approved by the Human Research Ethics Committee and by the Animal Care and Ethics Committee of the University of New South Wales.

Statistical Analysis

Data are expressed as means \pm SE and analyzed with repeated measures ANOVA (31). Fisher's protected least significant difference test was used for comparison of individual groups provided the *F* test was significant (31, 32). The Student *t* test was used for paired data (31).

Results

Orthotopic Model of Pancreatic Cancer

The survival rate in our model was 100%. No palpable tumors were detected in the abdomen of mice injected with PBS (group 1) or hPSCs alone (group 2). The latter observation suggests that hPSCs themselves do not have tumorigenic potential. Palpable abdominal tumors were first detected at \sim 4 weeks after the initial injection in 7 of 10 mice injected with MiaPaCa-2 + hPSCs (group 4) compared with only 2 of 10 mice injected with MiaPaCa-2 alone (group 3). All tumors increased in size over the next 3 weeks.

Macroscopic appearance of tumors and tumor volume. At 7 weeks, pancreas of group 1 and 2 mice showed no macroscopic

abnormality. Importantly, no tumors were observed at the site of injection in the pancreas of mice receiving hPSCs alone. In contrast to groups 1 and 2, all mice in groups 3 and 4 exhibited a tumor at the site of injection of cells in the tail of the pancreas (Fig. 1A). In general, tumors appeared as firm, grayish white single nodules easily distinguishable from the rest of the pancreas. One mouse from group 3 and two mice from group 4 exhibited a cluster of three to four tiny nodules at the site of injection. These nodules were in close contact with each other and, for the purposes of measurement of tumor size, were considered together as a single mass. In the majority of experimental sets (8 of 10 sets), the mouse injected with both MiaPaCa-2 and hPSCs (group 4) exhibited a larger tumor than its corresponding pair (i.e., the mouse injected with MiaPaCa-2 cells alone); of the remaining two sets, one pair had similar tumor volumes whereas the other showed a larger tumor in the MiaPaCa-2 injected mouse (Fig. 1B). Importantly, when the fold change in tumor volume of group 4 mice over their corresponding pairs injected with MiaPaCa-2 alone (group 3 mice/controls) was analyzed for all data points ($n = 10$ per group), there was a statistically significant increase in tumor volume in group 4 versus group 3 (Fig. 1B). These findings suggest that the presence of hPSCs induced the growth of pancreatic cancer in group 4 mice.

Microscopic appearance of pancreatic tumors. In pancreatic sections from group 3 and 4 mice, tumor cells were surrounded by normal mouse pancreatic acinar cells, confirming the orthotopic nature of the tumors (Fig. 1C). Within the tumor tissue of group 4

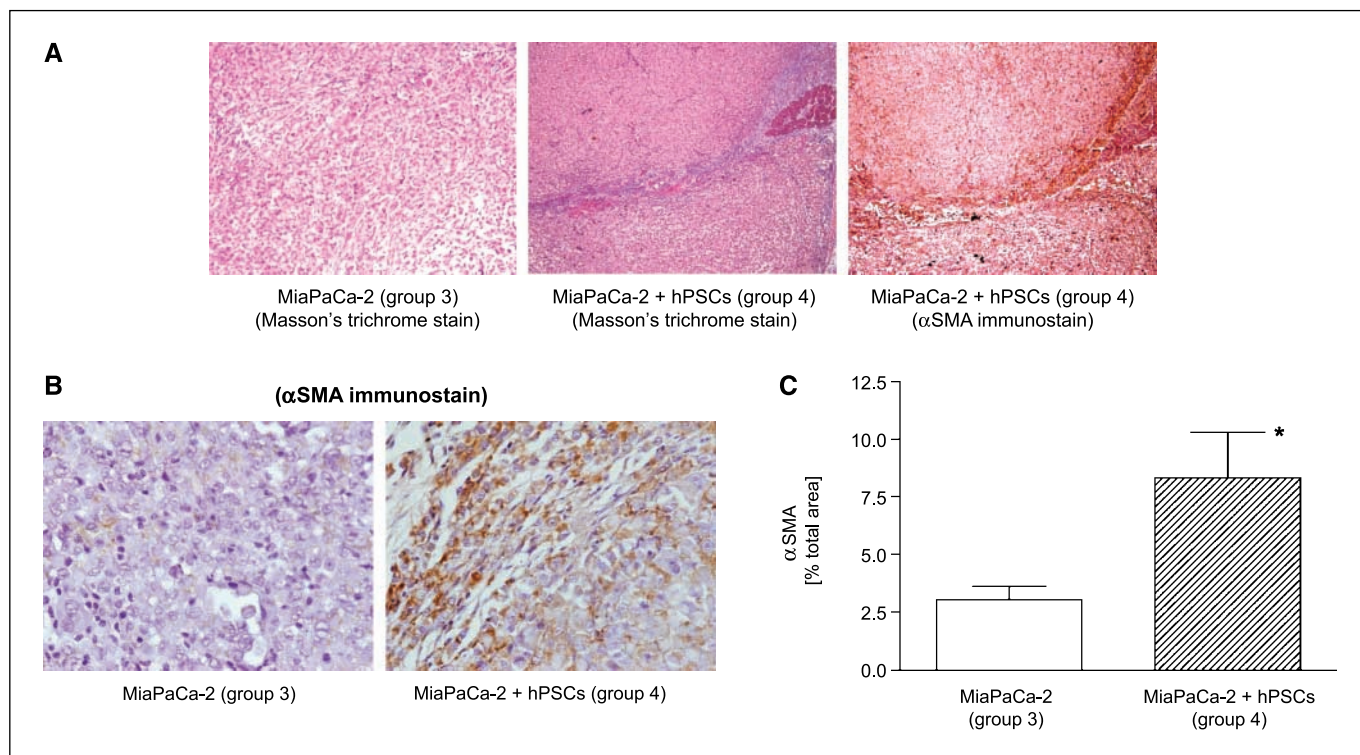


Figure 2. A, representative sections of Masson's trichrome staining and α SMA immunostaining of orthotopic pancreatic tumor sections. Representative sections from mice injected with MiaPaCa-2 cells alone (group 3) or MiaPaCa-2 + hPSCs (group 4). No positive Masson's trichrome staining can be seen in the group 3 tumor, indicating an absence of fibrous tissue. In contrast, a clearly delineated blue band of fibrosis can be seen in the group 4 tumor. A serial section of this tumor immunostained for α SMA shows close correlation between the areas staining blue (Masson's trichrome) and brown (α SMA), indicating the presence of activated PSCs in the fibrotic areas within the tumor (original magnification, $\times 200$). B, representative sections of orthotopic pancreatic tumors from mice injected with MiaPaCa-2 cells alone (group 3) or MiaPaCa-2 + hPSCs (group 4), immunostained for α SMA. Group 3 tumor shows minimal α SMA staining in a few discrete cells within the tumor tissue. In contrast, significant α SMA staining (indicating the presence of abundant activated PSCs) is observed throughout the group 4 tumor (original magnification, $\times 600$). C, morphometric analysis of all α SMA-stained sections indicated a significant increase in α SMA staining in group 4 compared with group 3 (*, $P < 0.02$; $n = 10$ animals per group).

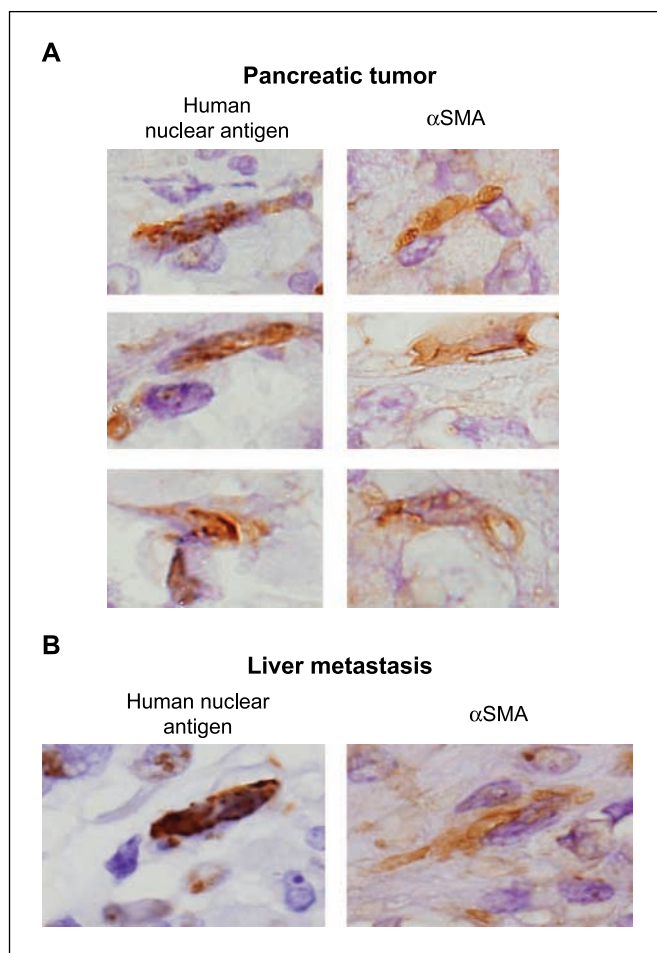


Figure 3. A, serial sections of three orthotopic pancreatic tumors from mice injected with MiaPaCa-2 + hPSCs immunostained for α SMA and antihuman nuclear antigen. Each α SMA-stained cell also exhibits positive staining for human nuclear antigen (original magnification, $\times 1,000$). B, serial sections of a metastatic liver nodule immunostained for α SMA and human nuclear antigen, showing an activated PSC (α SMA-positive) that also stains positive for human nuclear antigen (original magnification, $\times 1,000$).

mice, bands of fibrosis/stromal tissue were observed by both H&E (Fig. 1D) and Masson's trichrome (Fig. 2A) staining. Morphometric analysis revealed significantly increased Masson's trichrome staining in group 4 tumors compared with group 3 tumors (group 4: 9.06 ± 1.98 ; group 3: 3.22 ± 0.79 ; $P < 0.02$; $n = 10$ mice per group; data expressed as percent of total area). Notably, α SMA-positive cells were observed within the fibrous bands in the tumors of group 4 mice (Fig. 2B), confirming the presence of activated PSCs.

Interestingly, although none of the tumors in group 3 mice exhibited obvious fibrous tissue, faintly positive α SMA staining was observed in discrete cells within some tumors (Fig. 2B). These α SMA-positive cells may represent recruitment and activation of host (mouse) PSCs by the implanted human cancer cells. However, the extent of α SMA staining in group 3 tumors was significantly lower than that in group 4 tumors, as assessed by morphometry (Fig. 2C).

To assess whether the activated PSCs within the tumors in group 4 mice were a mixture of hPSCs and recruited host PSCs, we stained serial sections of group 4 tumors for α SMA and for the human cell-specific antigen, human nuclear antigen. These studies

showed that whereas several α SMA-positive cells also expressed the human nuclear antigen (i.e., these cells were hPSCs; Fig. 3A), there were some α SMA-positive cells that did not stain for the human-specific nuclear antigen. These observations suggest that whereas hPSCs survive within the tumor for the observation period, some recruitment of host (mouse) PSCs by pancreatic cancer cells also occurs.

Morphometry of cytokeratin staining in pancreatic sections from groups 3 and 4 revealed that the number of cancer cells per unit area (mm^2) of tumor was significantly higher in group 4 (group 4: 826 ± 36.31 ; group 3: $689 \pm 0.31.54$; $P < 0.0001$; $n = 10$ mice per group). Given that tumors in group 4 mice were significantly larger than those in group 3, the estimated total number of cancer cells per tumor in group 4 mice would be considerably higher than in group 3. This is an important observation because it indicates that the larger tumor size in group 4 cannot be attributed merely to the presence of fibrous tissue but is most likely predominantly due to increased cancer cell numbers. Increased numbers of proliferating cancer cells in group 4 tumors compared with group 3 tumors were confirmed by morphometric analysis of PCNA-stained sections (group 4: 38.2 ± 5.73 ; group 3: 21.6 ± 4.6 ; $P < 0.05$; $n = 10$; data expressed as percent of positive grid points). These findings support the concept that PSCs interact with cancer cells to promote tumor growth via an increase in cancer cell proliferation. Such an epithelial-stromal interaction has been confirmed by our *in vitro* studies (see below).

To determine whether reduced apoptosis could also contribute to the increased cancer cell numbers observed by cytokeratin staining, TUNEL staining was done. Our results indicate a trend toward lower numbers of TUNEL-positive cancer cells (suggesting less apoptosis) in group 4 tumors compared with group 3 tumors (0.97 ± 0.42 versus 3.27 ± 1.53 , respectively; $P = 0.12$; $n = 10$; data expressed as percent of positive grid points).

Regional and distant spread of pancreatic cancer. A significantly higher proportion of animals in group 4 than in group 3 exhibited tumor nodules in the mesentery (10% versus 0%), spleen (80% versus 20%), stomach (20% versus 0%), retroperitoneum (30% versus 20%), and abdominal wall (20% versus 10%). Whether these tumors can be considered true regional metastases (transcoelomic spread) or are the result of cancer cell spills during injections may be questioned. However, the latter possibility is relatively unlikely because some of the nodules were observed at sites that were left undisturbed during the injections.

Notably, distant metastasis was observed in the livers of 5 of 10 (50%) animals of group 4 compared with only 1 of 10 (10%) animals in group 3. These nodules were often multiple and located on the liver surface just underneath the liver capsule. H&E staining of liver nodules revealed the presence of cancer cells similar to those seen in pancreatic tumors. Interestingly, serial sections of metastatic liver nodules also showed the presence of α SMA-positive cells that were positive for human nuclear antigen (Fig. 3B), suggesting that spread of pancreatic cancer to distant sites may involve not only cancer cells but also stromal cells. The above observations further support the notion that the presence of hPSCs facilitates local and distant spread of pancreatic cancer.

Interaction of Pancreatic Cancer Cells and PSCs

Effect of pancreatic cancer cells on PSC migration. Migration of PSCs through the porous membrane of modified Boyden chambers was significantly greater in the presence of MiaPaCa-2 (2.3-fold) and Panc-1 (2-fold) than in their absence (Fig. 4A).

Effect of PSCs on pancreatic cancer cell migration and invasion. Migration of Panc-1 and MiaPaCa-2 cells was significantly increased in the presence of hPSCs (5.9- and 2.7-fold, respectively, at 48 hours; Fig. 4B). In addition, exposure of

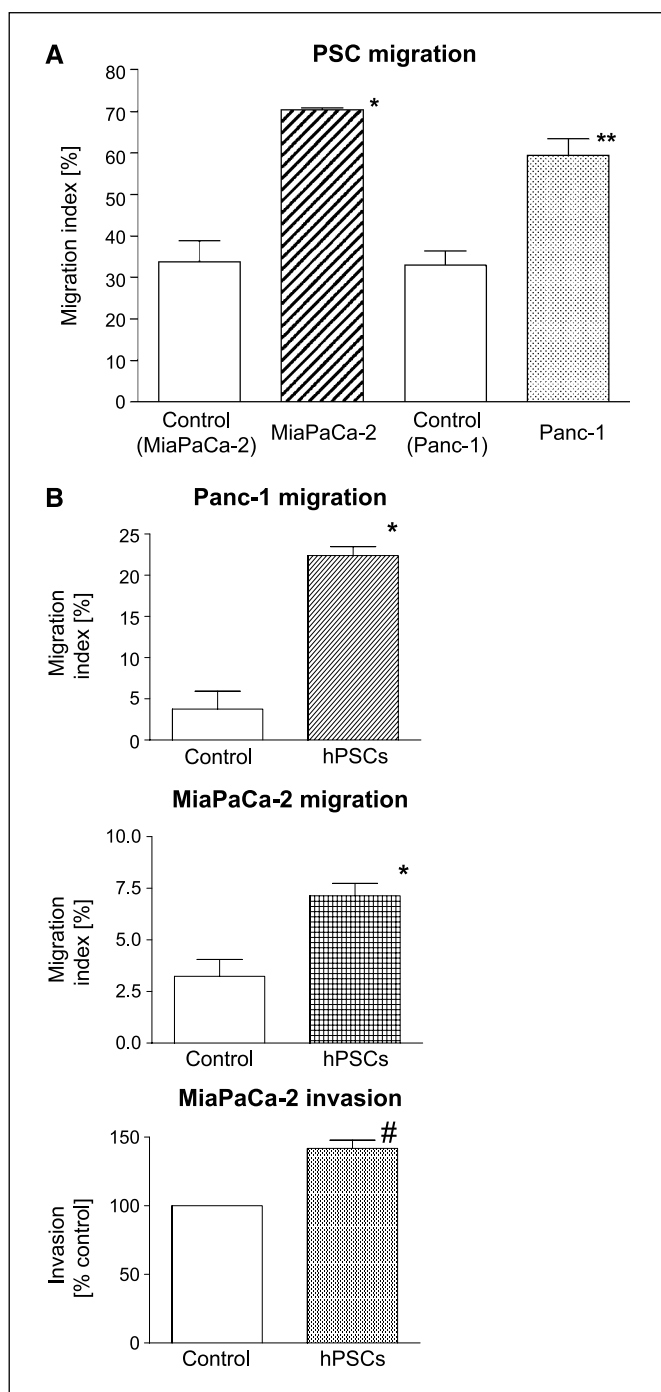


Figure 4. In the presence of MiaPaCa-2 or Panc-1, PSCs exhibited a significantly increased rate of migration compared with PSCs not exposed to pancreatic cancer cells. *, $P < 0.001$; **, $P < 0.002$, versus control. $n = 4$ separate PSC preparations. In the presence of hPSCs, migration of Panc-1 ($n = 4$ separate hPSC preparations) and MiaPaCa-2 ($n = 6$ separate hPSC preparations) cells exhibited significantly increased migration compared with cancer cells not exposed to hPSCs. *, $P < 0.002$, versus control. In addition, invasion of MiaPaCa-2 cells was significantly increased on exposure to hPSC secretions. #, $P < 0.01$. $n = 4$ separate hPSC secretions.

MiaPaCa-2 cells to hPSC secretions significantly enhanced their invasiveness as assessed by migration through basement membrane extract-coated porous membranes (Fig. 4B).

Effect of PSCs on pancreatic cancer cell proliferation. Proliferation of both MiaPaCa-2 and Panc-1 cells was significantly increased on exposure to PSC secretions for 24, 48, and 72 hours, compared with relevant controls (Fig. 5A).

Mediators of PSC-induced cancer cell proliferation. PSC secretion-induced MiaPaCa-2 proliferation was significantly inhibited by the neutralizing antibody to PDGF (Fig. 5B), but not by anti-TGF- β 1 or anti-bFGF antibodies (data not shown). These data suggest that PDGF in PSC secretions mediates, at least in part, the cancer cell proliferation observed in our experiments. This notion is not unreasonable given that PSCs express PDGF (Fig. 5C) and MiaPaCa-2 cells express the receptor for PDGF both at the mRNA level (as previously published; ref. 33) and, as we have found in this study, at the protein level (Fig. 5C).

Effect of PSCs on pancreatic cancer cell apoptosis. Human PSC secretions significantly inhibited both basal and H_2O_2 -induced apoptosis of MiaPaCa-2 and Panc-1 cells as assessed by TUNEL staining (Fig. 6A and B). Annexin V/propidium iodide studies confirmed that PSC secretions inhibited H_2O_2 -induced apoptosis of both MiaPaCa-2 and Panc-1 cells [data expressed as percent of H_2O_2 -stimulated apoptosis (mean \pm SE): MiaPaCa-2 + hPSC secretions, 50.85 ± 10.46 ($P < 0.01$; $n = 4$ separate hPSC secretions); Panc-1 + hPSC secretions, 52.95 ± 2.59 ($P < 0.01$; $n = 4$ separate hPSC secretions)].

Discussion

Using a novel approach to the production of orthotopic tumors in the pancreas, we have shown for the first time that an interaction exists *in vivo* between tumor and stromal cells, such that the presence of stromal cells significantly enhances pancreatic tumor growth rate as well as regional and distant metastasis. In our study, mice injected with a mixture of pancreatic cancer cells and hPSCs into the tail of the pancreas (group 4) exhibited significantly larger tumors within the gland compared with mice injected with cancer cells alone (group 3). No tumors were found in mice injected with hPSCs alone, suggesting that PSCs per se do not have tumorigenic potential. Importantly, the incidence of distant metastasis (liver nodules) was significantly higher in mice injected with both MiaPaCa-2 cells and hPSCs (50%) compared with those injected with MiaPaCa-2 alone (10%). In support of the above *in vivo* observations, our *in vitro* studies indicated that hPSC secretions increased pancreatic cancer cell proliferation and migration/invasiveness and, at the same time, inhibited cancer cell apoptosis. The net result of such an effect of hPSCs on pancreatic cancer cells would be a significant increase in the survival of cancer cells, which may explain, at least in part, our findings of significantly larger tumors in mice receiving both MiaPaCa-2 and hPSCs.

Although it could be argued that the increased tumor size in group 4 was predominantly due to increased fibrous/stromal tissue within the cancer, histologic analyses with Masson's trichrome, cytokeratin, and PCNA stains indicated that this was not the case. Morphometric evaluation of fibrosis (Masson's trichrome staining) versus tumor cell numbers (cytokeratin staining) showed that fibrosis accounted for only a minor proportion of tumor volume. The bulk of the tumor was composed of tumor cells themselves. PCNA staining of tumor sections confirmed increased number of

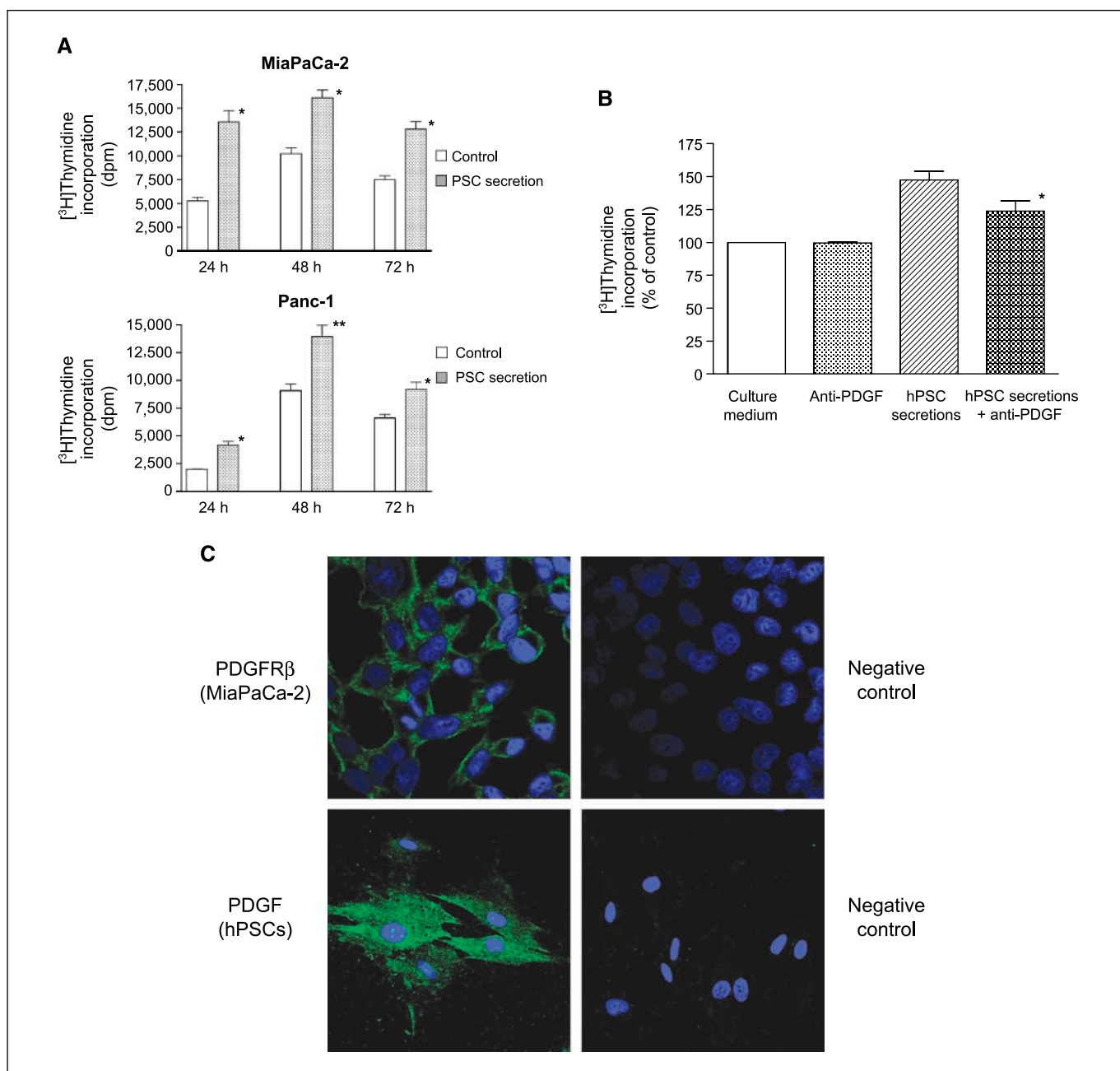


Figure 5. A, both MiaPaCa-2 cells and Panc-1 cells exhibited significantly increased proliferation when exposed to conditioned medium from hPSCs for 24, 48, and 72 h, compared with control cells incubated with culture medium alone. *, $P < 0.005$; **, $P < 0.03$. $n = 5$ separate hPSC preparations. B, compared with controls (cells incubated in culture medium alone or in medium + anti-PDGF antibody in the absence of secretions), proliferation of MiaPaCa-2 cells was induced by hPSC secretions. This proliferative effect was significantly inhibited in the presence of a PDGF neutralizing antibody. *, $P < 0.03$. $n = 3$ separate hPSC secretions. C, hPSCs and MiaPaCa-2 cells exhibited immunoreactivity for PDGF (original magnification, $\times 600$) and PDGF receptor β ($PDGFR\beta$; original magnification, $\times 1,000$), respectively.

proliferating cancer cells in group 4 compared with group 3, supporting the notion that tumor cell proliferation is stimulated in the presence of hPSCs. Interestingly, TUNEL staining of tumor sections showed a trend toward reduced apoptosis of cancer cells in group 4 versus group 3 tumors. Thus, our *in vivo* studies suggest improved survival (secondary to increased proliferation and, possibly, decreased apoptosis) of cancer cells in the presence of PSCs. These *in vivo* findings concur with the results of our *in vitro* studies (see below).

As expected, in group 4 tumors, activated PSCs were found in areas of fibrosis as well as discrete cells dispersed throughout the tumor. However, of interest was the finding of some α SMA-positive cells in tumors of group 3 mice, suggesting recruitment of host (mouse) PSCs by the exogenous tumor cells and supporting the concept that pancreatic tumor cells influence stromal cell function to establish a growth-permissive environment that facilitates progression of the disease. It is important to note, however, that there was a significant difference in the extent of PSC activation

between group 3 and group 4 tumors, suggesting the following possibilities: (a) the presence of activated hPSCs facilitates recruitment of mouse PSCs; (b) the presence of activated hPSCs influences cancer cell function such that the latter become more effective recruiters of host stromal cells. Indeed, as discussed below, our *in vitro* studies have shown that pancreatic cancer cells induce PSC migration.

An important observation in our model was increased incidence of distant (liver) and regional metastases in group 4 compared with group 3, suggesting that stromal cells stimulate or facilitate regional and distant invasion of pancreatic tumors. The finding of human nuclear antigen and α SMA-positive cells in liver nodules raises an intriguing question: Are metastatic tumor cell clusters that originate in the pancreas accompanied by PSCs to distant sites? To confirm our observations, it will be necessary to transfect hPSCs with an identifiable marker that can be traced *in vivo* after injection of the cells into the pancreas. These experiments are beyond the scope of our current study, but will form the basis of future studies in our laboratory.

The mechanisms mediating the facilitatory effect of PSCs on tumor cells may be attributed to two known functions of PSCs: (a) secretion of matrix metalloproteinases enabling degradation of ECM and facilitating local tumor invasion (28), and (b) secretion of angiogenic factors such as TGF- β 1 and VEGF thereby stimulating neoangiogenesis (15, 18, 34). Of particular relevance to the latter point is the recent work by Friess et al. (35, 36) showing that increased expression of TGF β and VEGF in stromal areas of pancreatic cancer is associated with poor patient outcome.

Results of our *in vitro* studies support the interaction observed *in vivo* between cancer cells and PSCs. We and others have previously established that pancreatic cancer cells stimulate PSC proliferation and activation. In the present study, we have shown that pancreatic cancer cells also stimulate PSC migration, an effect that may be mediated by PDGF because (a) PDGF is secreted by cancer cells; (b) PDGF receptor expression is induced in activated PSCs (20); and (c) PDGF exerts a chemotactic effect on PSCs (37). Interestingly, we have also found that PSCs in turn increase cancer cell migration, invasion, and proliferation. The observed increase in migration and invasion suggests that PSCs increase local aggressiveness of cancer. The increased proliferation was of particular interest because it indicates that cancer cells, despite a high basal proliferative rate, can be further induced to proliferate in the presence of factors (e.g., PDGF) secreted by activated PSCs.

Resistance to apoptosis is a feature of several tumor cell types and is thought to be one of the mechanisms of acquired resistance to chemotherapeutic agents (38, 39). Our current studies indicate that PSC secretions inhibit apoptosis of pancreatic cancer cells. However, it may be speculated that this effect is mediated by the ECM proteins laminin and fibronectin secreted by PSCs, given the recent report that these proteins inhibit apoptosis of pancreatic cancer cells *in vitro* (40). Given that PSCs are known to secrete ECM proteins, it may be speculated that the observed inhibition of cancer cell apoptosis in response to PSC secretions may be mediated via proteins such as laminin and fibronectin in the conditioned medium.

In conclusion, this study is the first to provide evidence of the facilitatory effect of PSCs on pancreatic cancer progression within

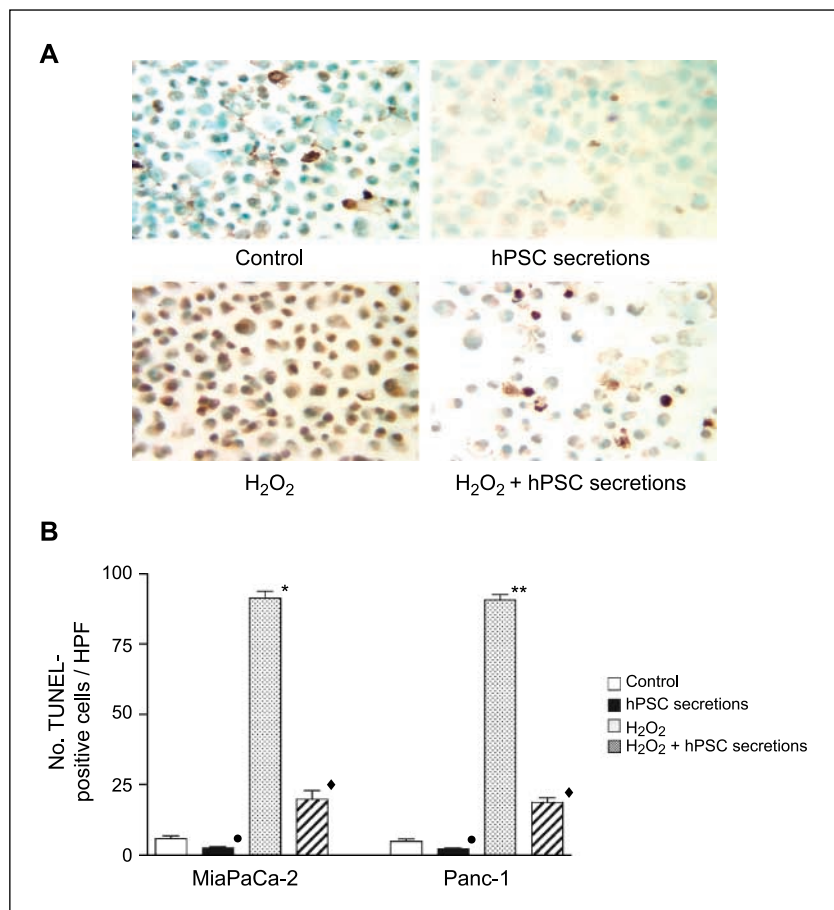


Figure 6. A, TUNEL staining of a representative set of cytopins of MiaPaCa-2 cells treated with culture medium alone (control), H₂O₂, hPSC secretions, or H₂O₂ + hPSC secretions. Basal apoptosis (brown TUNEL-positive staining) in control cells was significantly increased by H₂O₂. In the presence of PSC secretions, basal as well as H₂O₂-induced apoptosis was significantly reduced. Similar results were observed with Panc-1 cells. B, morphometric analysis of TUNEL-stained cytopins. *, $P < 0.01$; **, $P < 0.005$, H₂O₂-induced versus basal apoptosis; ♦, $P < 0.005$, hPSC secretions versus basal apoptosis; ♦, $P < 0.01$, H₂O₂ + hPSC secretions versus H₂O₂-induced apoptosis. $n = 3$ separate hPSC secretions.

the organ of interest as well as to distant sites. We have provided evidence of a bidirectional interaction between tumor cells and stromal cells. Our findings support the concept that pancreatic cancer cells recruit PSCs to their immediate vicinity to establish a growth-supportive and growth-permissive environment. Therapeutic targeting of stromal cells and/or strategies to interrupt the tumor-stromal cell interaction in pancreatic cancer may represent an important therapeutic approach in this aggressive disease.

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